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## Control of Prolactin Secretion in the Teleost *Oreochromis mossambicus*: Effects of Water Acidification

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Prolactin secretion is stimulated markedly in fish exposed to water of pH 4. This phenomenon was used to study the control of prolactin secretion. Activation occurs irrespective of changes in plasma osmolarity or plasma sodium and calcium concentrations. After acute acidification of the water, which leads to a substantial fall in plasma osmolarity and plasma electrolyte levels, the activation of the prolactin cells is less marked than after gradual acidification of the water, when plasma osmolarity, plasma sodium, and plasma total and ionic calcium levels are not noticeably affected. When fish bearing an implanted rostral pars distalis of the pituitary gland are exposed to water of pH 4, both the *in situ* prolactin cells and the prolactin cells of the implant become activated only when the drop in water pH is acute and followed by a reduction in plasma osmolarity and electrolyte levels. When the rate of reduction of the pH is slow and not changing plasma osmolarity or sodium and calcium levels, the *in situ* prolactin cells are stimulated, but not those of the implants. We conclude that the activation of the prolactin cells *in situ* in fish in acid water is not mediated by reductions in plasma osmolarity, plasma sodium, total calcium, or ionic calcium, but by hypothalamic mechanisms. The drop in plasma osmolarity and electrolytes probably reflects excessive osmoregulatory stress and this may hamper instead of stimulate the response of the prolactin cells to osmoregulatory disturbance. The physiological significance of the *in vitro* activation of prolactin cells by reduced ambient osmolarity is unclear. © 1988 Academic Press, Inc.

The secretory activity of the prolactin cells in fish is influenced by the osmolarity and ionic content of the ambient water. In seawater fish, prolactin secretion and prolactin blood levels are low. In freshwater fish, prolactin secretion as well as circulating prolactin levels are generally high (Clarke and Bern, 1980; Nicoll *et al.*, 1981; Prunet and Boeuf, 1985; Hirano, 1986). The high rate of prolactin secretion of freshwater fish has been ascribed to the osmolarity or the sodium or calcium and magnesium concentrations of the water, which are extremely low when compared with those of seawater (Ensor and Ball, 1972; Nagahama *et al.*, 1974, 1975; Ogasawara and Yamada, 1979; Wendelaar Bonga and Van der Meij, 1980, 1981; Olivereau and Olivereau, 1983; Ruijter *et al.*, 1984).

Recently, we have shown that in some

species reduction of water pH also results in marked stimulation of prolactin secretion (Wendelaar Bonga *et al.*, 1984a, b). Reductions of water osmolarity, calcium concentration, or water pH all induce increased electrolyte losses that may result in reduction of plasma osmolarity and plasma electrolytes, and in increased osmotic water uptake. These losses are probably counteracted by enhanced prolactin secretion.

The mechanisms that mediate the effects of water osmolarity and ionic concentrations on prolactin cells are still under debate. Neuroendocrine as well as systemic pathways have been implicated. As in terrestrial vertebrates, aminergic and peptidergic neural mechanisms are known to influence prolactin secretion in fish (Ball, 1981). On the other hand, it has been concluded that the osmolarity of the water af-



fects prolactin cells more directly, via changes induced in the osmolarity of the blood. This was based on observations on fish bearing ectopically grafted pituitary glands and on incubated or perfused pituitary glands. When fish bearing grafted pituitary glands are transferred from freshwater to seawater, the prolactin cells are inactivated, similar to prolactin cells *in situ*. This effect has been ascribed to the inhibitory effect on these cells of increased plasma osmolarity (Leatherland and Ensor, 1973; Nagahama *et al.*, 1974). Similarly, when prolactin cells *in vitro* are exposed to an increase in the osmolarity of the incubation or the perfusion fluid, synthesis and release of prolactin are reduced, whereas the reverse is found after reduction of the osmolarity (Ingleton *et al.*, 1973; Nagahama *et al.*, 1975; Wigham *et al.*, 1977; Grau *et al.*, 1981, 1987; Batten *et al.*, 1983). We have confirmed these observations for prolactin cells of *Oreochromis mossambicus in vitro* (Wendelaar Bonga *et al.*, 1985). However, additional experiments indicated that the response to ambient osmolarity of prolactin cells *in vitro* or in pituitary grafts does not always occur in prolactin cells *in situ*, i.e., in the presence of intact hypothalamic connections. In these experiments we observed that the reduction of plasma osmolarity following exposure of fish to calcium-deficient fresh water stimulates prolactin secretion, whereas a similar reduction effected by exposure to high-calcium water reduces prolactin secretion. Thus, consistent activation of prolactin cells by reduced osmolarity seems to occur only in the absence of hypothalamic control mechanisms.

In the present study we have examined the relationship between plasma osmolarity and prolactin secretion in fish during exposure to acid water. Prolactin secretion is increased dramatically in water of pH 4 (Wendelaar Bonga *et al.*, 1984a, b). This is often preceded by a reduction in plasma osmolarity. However, the drop in osmolarity

is dependent on the rate of acidification of the water, rather than on the final water pH (Wendelaar Bonga *et al.*, 1987). We compared the response to water acidification of the prolactin cells of *O. mossambicus in situ* and in ectopic pituitary implants. Water pH was reduced to pH 4 either abruptly, which resulted in an appreciable loss of plasma electrolytes and thus in reduction of osmolarity, or gradually, without noticeable reduction of plasma osmolarity. Since plasma sodium and calcium have been implicated in the control of prolactin secretion in addition to osmolarity, both were studied to examine the relationship between these parameters and the synthetic activity of the prolactin cells *in situ* and in implants.

## MATERIALS AND METHODS

Sexually mature tilapia (*O. mossambicus*) of about 15 g body wt were used. The fish were obtained from laboratory stock and kept in 100-liter aquaria at 25° with a daily light period of 12 hr. The aquaria contained well-aerated circulating tapwater. The concentrations of the main ions of the water used were (in mM) Na<sup>+</sup>, 3.0; K<sup>+</sup>, 0.08; Ca<sup>2+</sup>, 0.8; Mg<sup>2+</sup>, 0.2; Cl<sup>-</sup>, 4.2; and SO<sub>4</sub><sup>2-</sup>, 0.5. The water pH was 7.6.

Three days before the water was acidified, pituitary glands were dissected from normal freshwater donor fish. The rostral pars distalis containing all the prolactin cells (prolactin lobe) was separated carefully from the pituitary gland. Three prolactin lobes per recipient fish were implanted via a small but deep incision (2–3 mm in length, about 8 mm deep) in the dorsal musculature. The recipient fish were anesthetized lightly with MS-222. The incision was sutured carefully. The control fish were treated similarly, but no tissue was implanted.

Water pH was reduced from pH 7.6 to pH 4 either abruptly (within 10 min) or gradually (over 4 hr) by adding diluted H<sub>2</sub>SO<sub>4</sub> (analytical grade; 0.05 M solution in tapwater) to the water circulation. Water pH was controlled by pH stat equipment (Radiometer, Copenhagen). The ammonia concentration of the water (ammonia secretion increases rapidly during adaptation to acid water) was monitored daily and kept below 0.5 mg per liter by refreshing part of the water.

Four, 16, 24, and 48 hr after the start of acidification of the water groups of fish were anesthetized rapidly in methoxyethanol. Fish bearing prolactin lobes were killed 48 hr after the start of acidification. Blood was collected from the cut end of the caudal peduncle in heparinized hematocrit capillaries. Blood samples



were analyzed for ionic calcium within 15 min after sampling in an ICA-I automated ionic calcium analyzer (Radiometer). After centrifugation of the blood, 50- $\mu$ l plasma samples were used for determination of the osmolarity in a Vogel microosmometer. Plasma  $\text{Na}^+$  was determined by atomic absorption spectrophotometry. Plasma calcium was determined by the use of a calcium kit (Sigma Chemical Co., St. Louis, MO), a colorimetric method based on cresolphthalein complexone.

For electron microscopy pituitary glands were fixed as described earlier (Wendelaar Bonga and Van der Meij, 1980). Ultrathin sections were examined in a Philips 201 or 301 electron microscope. The number of secretory granules per unit area of cytoplasmic surface (granule density) was determined with Kontron Digiplan integration equipment and a magnetostriction tablet. Randomly selected prolactin cell profiles containing a nucleus and with a total cytoplasmic area of 500  $\mu\text{m}^2$  per gland (nuclei excluded) were analyzed, at a final magnification of 8600 $\times$ .

To estimate the rate of prolactin synthesis, the rostral pars distalis (prolactin lobe) was carefully separated from freshly dissected pituitary glands. After a preincubation period of 30 min, the lobes were incubated (in batches of three lobes) for 4 hr in a medium containing [ $^3\text{H}$ ]leucine. Subsequently the lobes and incubation media were subjected to SDS-gel electrophoresis (for a detailed description of the procedures see Wendelaar Bonga *et al.*, 1983). In our system tilapia prolactin is represented by two bands of 20 and 21.5 kDa, respectively (Wendelaar Bonga *et al.*, 1984a), which likely represent the two bands described by Specker *et al.* (1985) for the same species, and shown to differ slightly in amino acid composition. In tilapia both bands show similar biological activity (Specker *et al.*, 1985) and they are synthesized and released in the same ratio under different conditions (Wendelaar Bonga *et al.*, 1984a; Specker *et al.*, 1985). Therefore, the rate of prolactin synthesis was estimated by summarizing the total amount of labeled (and thus newly synthesized during incubation) prolactin present in both bands, and recovered from both lobes and incubation media. The radioactivity present in the bands was quantified by autoradiography of the gels and subsequent densitometric scanning of the autoradiograms with a Bio-Rad Model 1650 densitometer. The results are expressed as percentage of the controls.

Differences between the experimental groups concerning granule density and plasma ion values were tested for significance by the Mann-Whitney *U* test.

## RESULTS

### 1. Plasma Osmolarity, Sodium, and Calcium

Acute acidification of the water to pH 4.0

led to a rapid reduction of plasma osmolarity, plasma sodium and plasma total calcium, which became noticeable within 1 hr (results not shown). The differences from the control values were highly significant at 4 hr (sodium and total calcium;  $P < 0.01$ ) and 16 hr (osmolarity;  $P < 0.001$ ) after the start of acidification, until the end of the exposure period (Fig. 1). The blood ionic calcium concentration was also reduced, from  $1.47 \pm 0.11 \text{ mM Ca}^{2+}$  in the controls, to  $1.21 \pm 0.09 \text{ mM Ca}^{2+}$  after 24 hr at pH 4 ( $P < 0.05$ ).

When the reduction of water pH was gradual, with the final pH reached after 4 hr, no reduction in plasma osmolarity or sodium and total calcium concentrations was observed (Fig. 1). Similarly, blood ionic calcium levels were not affected ( $1.51 \pm 0.12 \text{ mM Ca}^{2+}$  in the controls;  $1.46 \pm 0.08 \text{ mM Ca}^{2+}$  in fish exposed for 48 hr to pH 4).

### 2. Prolactin Cell Activity

**Ultrastructure.** Four hours after acute acidification of the water the ultrastructure of the prolactin cells was unchanged. However, after 16 hr a notable degranulation was observed (Figs. 1–3), probably caused by increased exocytosis of the secretory granules. Granule density remained low until the end of the observation period. During the first 24 hr no other structural changes were found. After 48 hr, however, Golgi areas were increased in extent. Whereas the secretory granules outside the Golgi area had decreased markedly, the number of presecretory granules present in the Golgi areas had increased.

When water pH was reduced slowly, the changes in the ultrastructure of the prolactin cells were similar to those observed after acute acidification (Fig. 4). The extent of degranulation was slightly more pronounced (Fig. 1). After 48 hr, the cells showed the same activation of the Golgi areas as after acute acidification (Fig. 4).



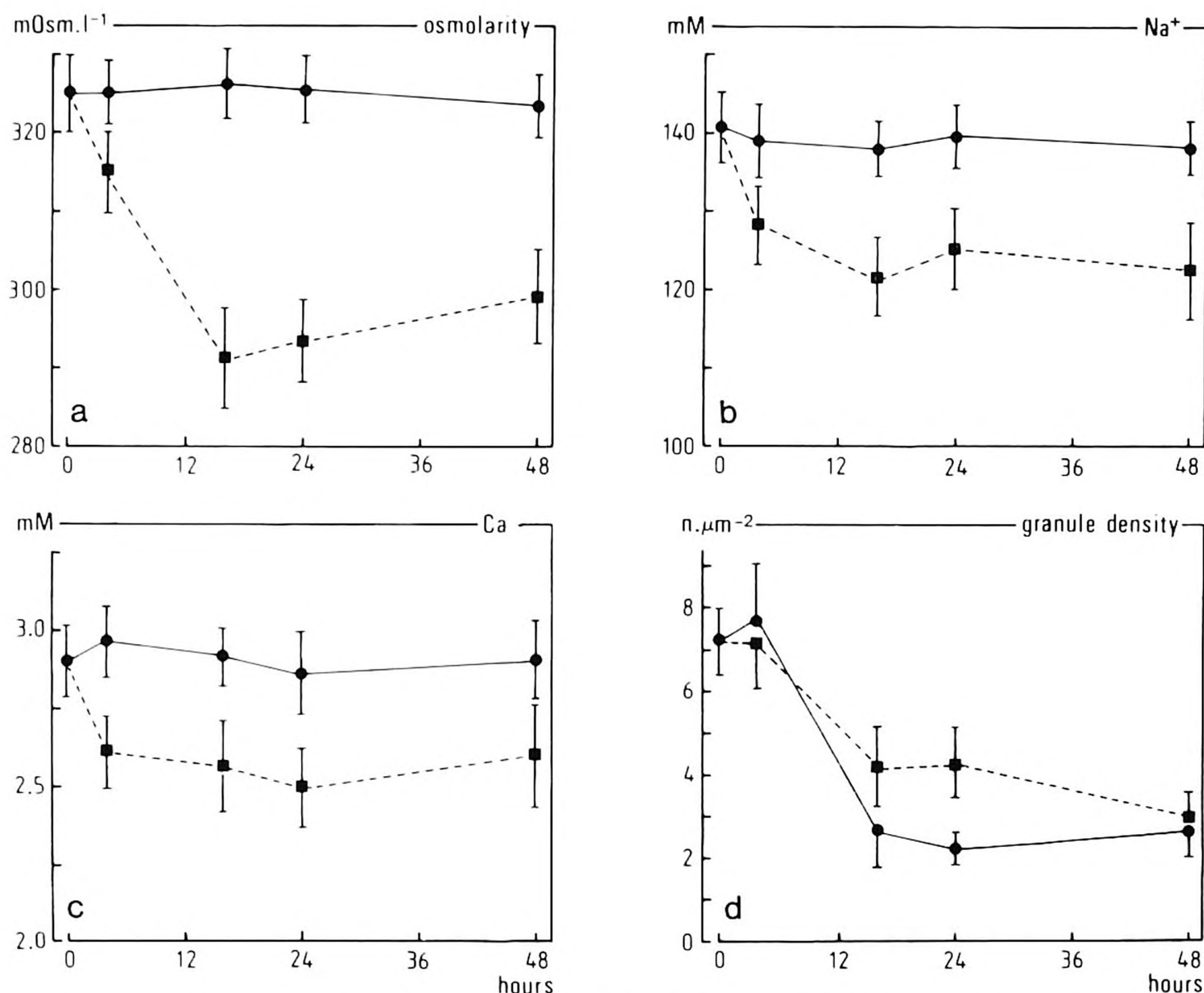


FIG. 1. Plasma osmolarity (a), sodium (b), total calcium (c), and density of the secretory granules of the prolactin cells (d) of fish after gradual water acidification (solid lines) or acute water acidification (broken lines); acidification started at  $t = 0$  hr. Granule density is expressed as the number of secretory granules per surface unit of cytoplasm as determined in electron micrographs; means  $\pm$  SD of seven (a, b, c) or five fishes per group.

**Prolactin synthesis.** To estimate the rate of prolactin synthesis, freshly dissected rostral lobes of the pituitary glands—these lobes contain all of the prolactin cells—were incubated *in vitro* in the presence of labeled amino acids. Forty-eight hours after acute acidification to pH 4.0, the rate of prolactin synthesis of the rostral lobes of acid-exposed fish was two times higher than that of control fish. The lobes of the fish experiencing slow acidification to pH 4 showed an almost threefold increase in the rate of prolactin synthesis after 48 hr ( $P < 0.01$ ; Fig. 5).

### 3. Implanted Prolactin Lobes

Rostral lobes of pituitary glands of donor fish were implanted in the dorsal muscula-

ture of acceptor fish that were subsequently exposed to pH 4 by acute water acidification. After 48 hr, plasma osmolarity was reduced significantly when compared to that of controls bearing implants that were not exposed to acid water (controls,  $315 \pm 6$  mOsm  $\cdot$  liter<sup>-1</sup>; pH 4,  $274 \pm 5$  mOsm  $\cdot$  liter<sup>-1</sup>;  $P < 0.01$ ). The prolactin cells *in situ* and those in the implants behaved similarly; both showed degranulation (Figs. 6 and 7).

When fish with implanted rostral lobes were exposed to gradual acidification of the water of pH 4, plasma osmolarity was similar to that of controls (controls,  $312 \pm 7$  mOsm  $\cdot$  liter<sup>-1</sup>; pH 4,  $315 \pm 9$  mOsm  $\cdot$  liter<sup>-1</sup>). The prolactin cells of the *in situ* pituitary glands were degranulated to



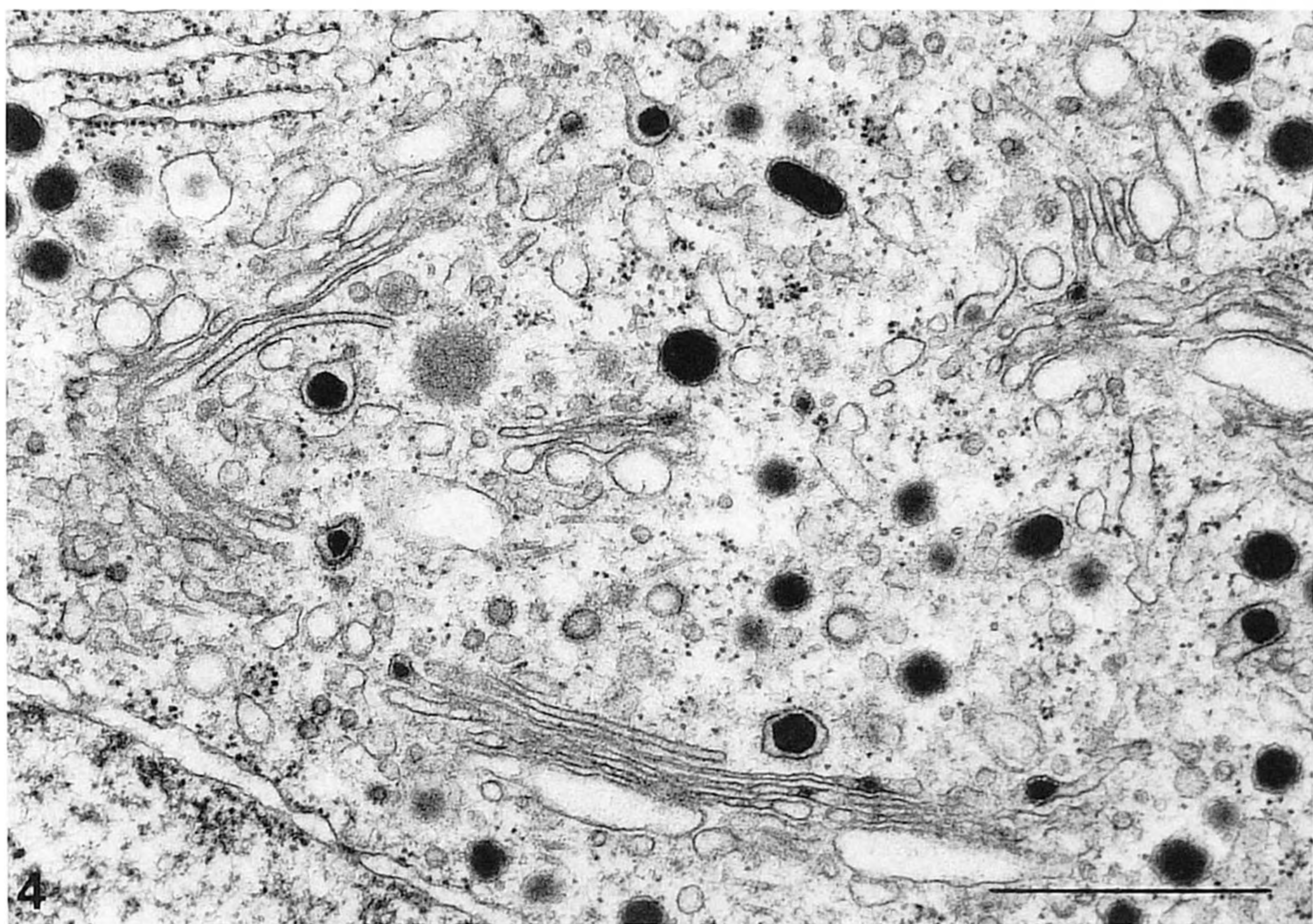
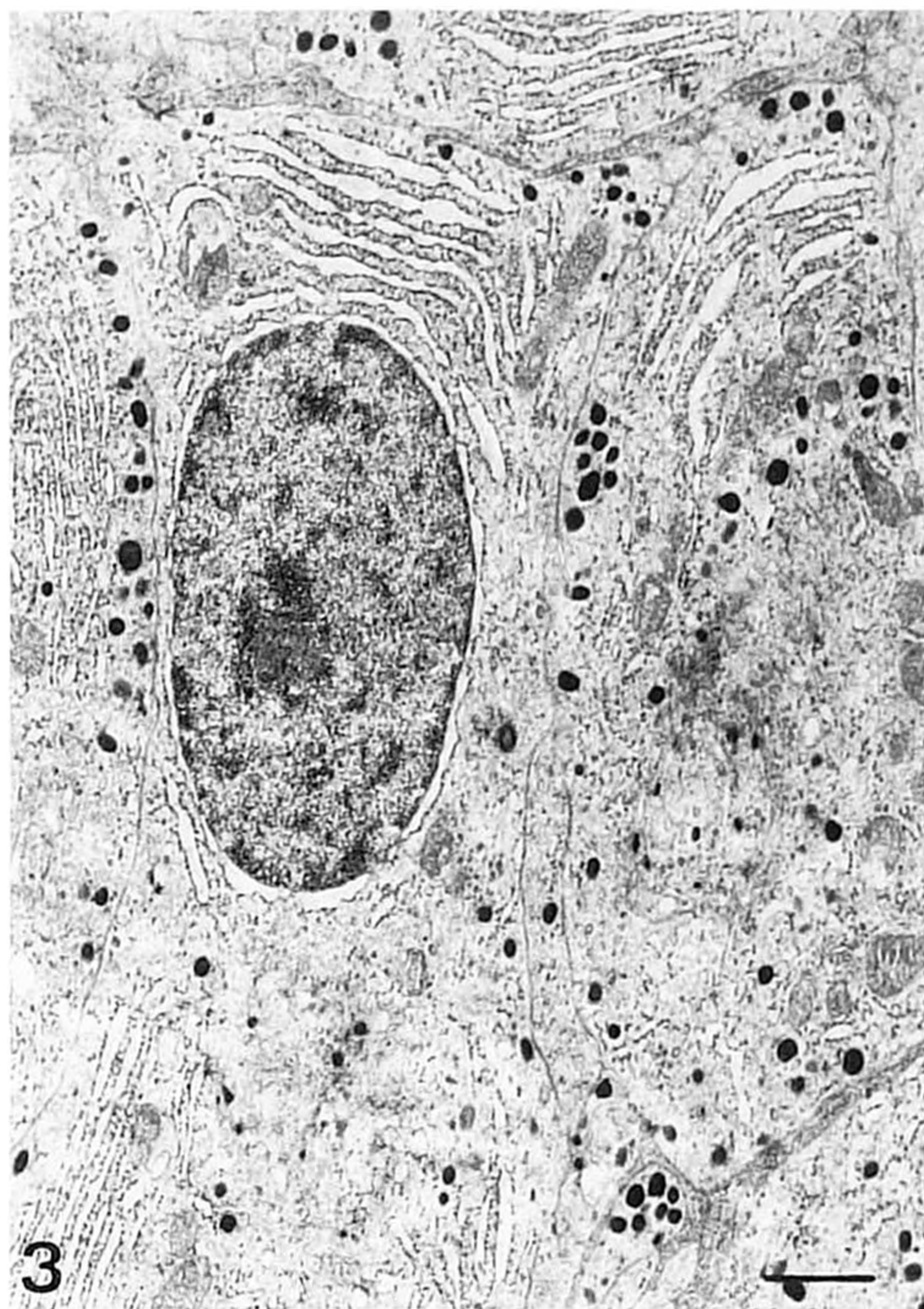
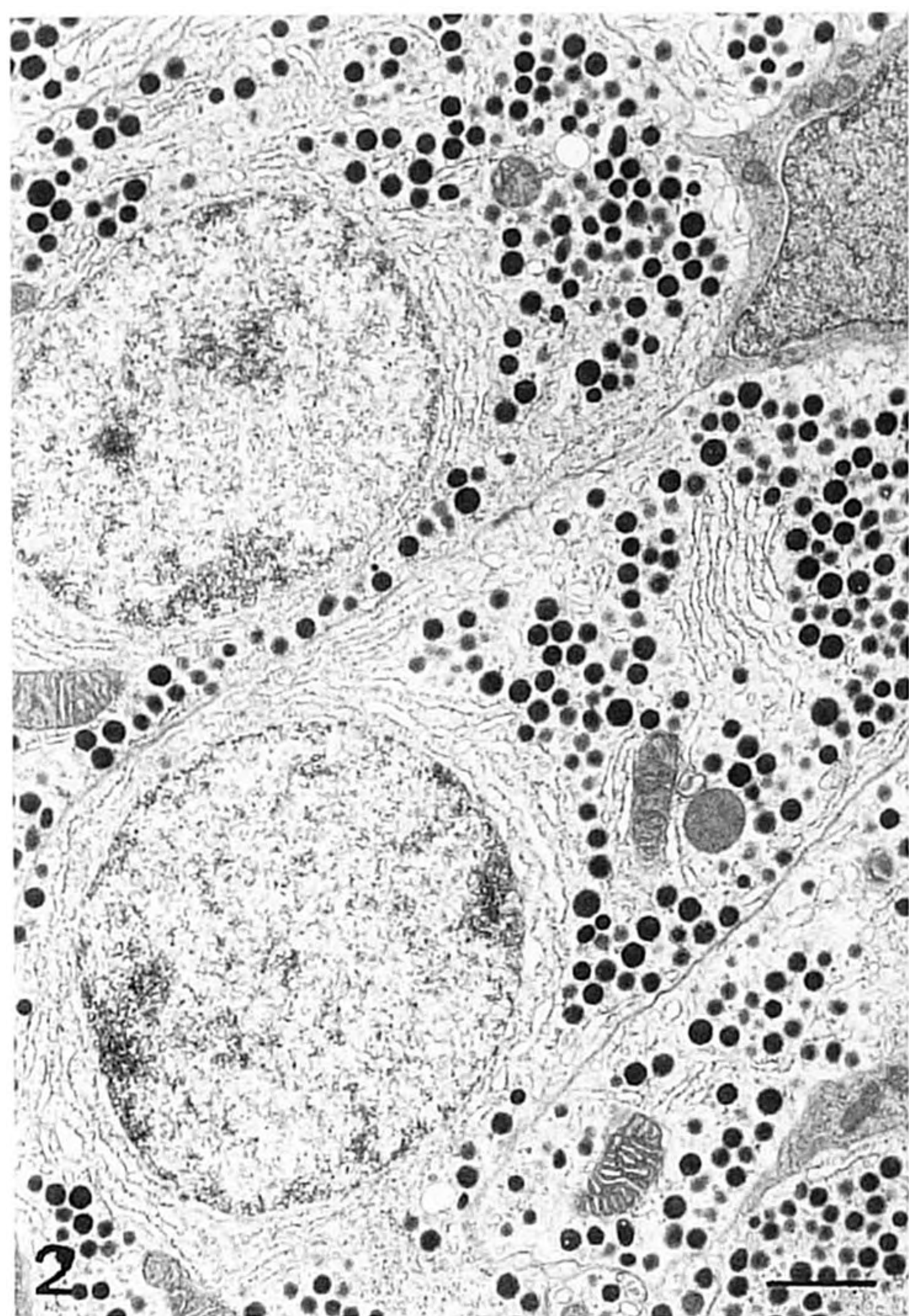


FIG. 2. Prolactin cell of *in situ* pituitary gland of control fish from neutral freshwater; bar represents 1  $\mu$ m.

FIG. 3. Degranulated prolactin cells of *in situ* pituitary gland of fish 16 hr after acute water acidification; bar represents 1  $\mu$ m.

FIG. 4. Active Golgi area of prolactin cell of *in situ* pituitary gland of fish exposed for 48 hr to acid water (gradual acidification); bar represents 1  $\mu$ m.



the same extent as described above for fish without implants. However, the prolactin cells of the implanted rostral lobes showed no degranulation (Figs. 6 and 8). The granule density was similar to that of implants in the control fish from neutral water ( $9.43 \pm 1.42$  per  $\mu\text{m}^2$ ).

## DISCUSSION

### *Prolactin Secretion and Water Composition*

Prolactin secretion is rapidly and significantly stimulated in tilapia exposed to water of pH 4. This is in agreement with our earlier results on the effects of prolonged acidification of the water in this species (Wendelaar Bonga *et al.*, 1984a, b, 1987). Increased prolactin secretion by water acidification is also indicated by a study of Notter *et al.*, (1976), who demonstrated an increase in RNA synthesis of the prolactin cell of trout exposed to acid water for 3–5 days. Our present report deals with the immediate effects of acidification in tilapia. Rapid degranulation of the prolactin cells occurs between 4 and 16 hr after the start of

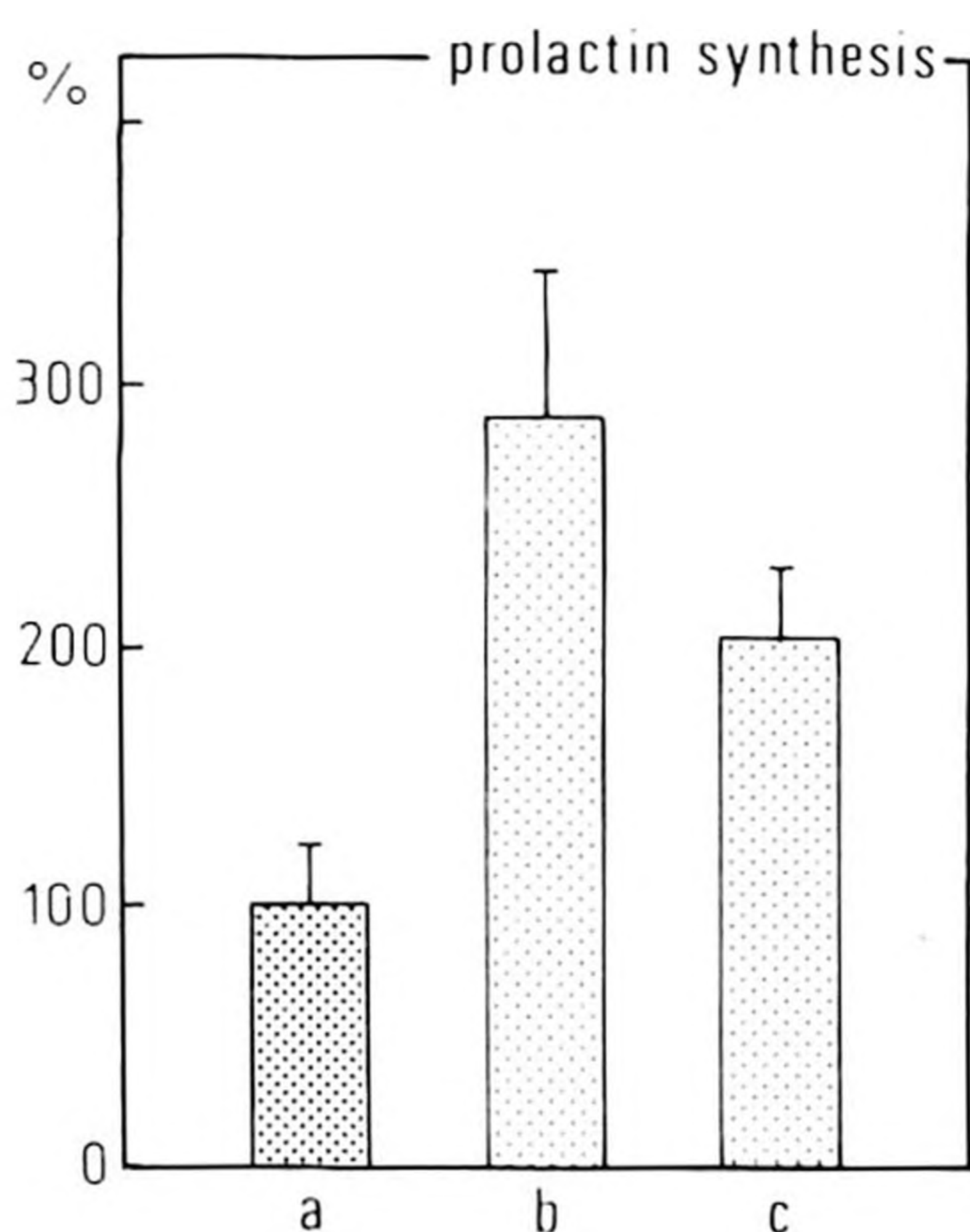


FIG. 5. Relative rate of prolactin synthesis of pituitary glands (means  $\pm$  SD;  $n = 5$ ) freshly dissected from control fish (a, water pH 7.6) or from fish exposed to acid water (b, gradual acidification; c, acute acidification).

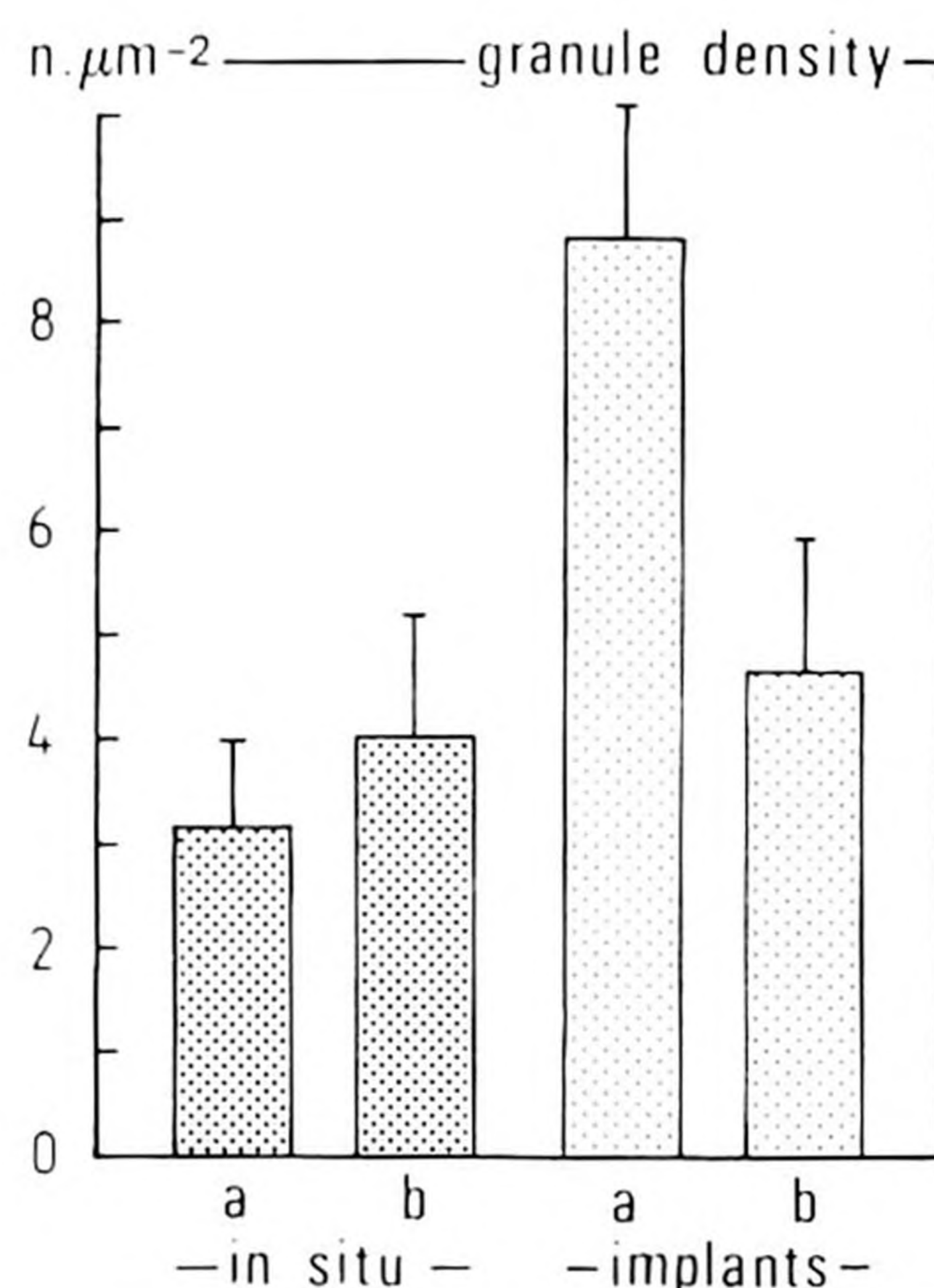


FIG. 6. Number of secretory granules per surface unit of cytoplasm, as determined in electron micrographs, of prolactin cells of *in situ* pituitary glands (left bars) or of pituitary glands implanted in the same fish (right bars), 48 hr after acidification of the water; a, gradual water acidification; b, acute water acidification (means  $\pm$  SD;  $n = 5$ ).

acidification. This indicates that during this period the rate of granule extrusion surpasses the rate of granule synthesis. A two- to threefold increased rate of prolactin synthesis could be demonstrated in prolactin lobes freshly dissected from fish exposed to pH 4 for only 48 hr. Thus, acidification of the water is an effective treatment to stimulate prolactin secretion in tilapia.

Factors other than water pH that have been reported to stimulate prolactin secretion in fish include reduction of the osmolarity and the calcium concentration of the water. Enhanced prolactin cell activity after reduction of water osmolarity, as occurs after transfer or migration of fish from seawater to freshwater, has been reported for many species (Ensor and Ball, 1972; Nagahama *et al.*, 1973; Wigham and Ball, 1977; Dubourg *et al.*, 1980; Olivereau *et al.*, 1981). The absence of such a stimulatory effect is exceptional (Cook and Van Overbeke, 1969). The effect of low water calcium levels has been studied in relatively few species. An inverse relationship be-



tween prolactin cell activity and the calcium concentration in freshwater has been reported for sticklebacks, tilapia, eels, and catfish (Wendelaar Bonga, 1978; Wendelaar Bonga and Van der Meij, 1980; Ogasawara and Yamada, 1979; Olivereau and Olivereau, 1983; Srivastav *et al.*, 1983). In goldfish changes in osmolarity, but not in water calcium concentration, influence prolactin secretion (Olivereau *et al.*, 1981). We have further shown that low concentrations of cadmium ions in water ( $10\ \mu\text{M}$ ) stimulate prolactin secretion in tilapia (Fu *et al.*, in preparation).

Treatments such as reduction in water osmolarity, calcium concentration, or pH, or the addition of cadmium to the water, have in common that they all enhance passive ion losses and osmotic water uptake across the integument, in particular the epithelium covering the gills (McDonald, 1983; Giles, 1984; Wendelaar Bonga *et al.*, 1985, 1987; Flik *et al.*, 1987; Hirano, 1986). The stimulation of prolactin following such treatments in tilapia and some other species can be considered as an appropriate response: the principal function of prolactin in freshwater osmoregulation seems to be the control of the permeability of the integument to water and ions (Hirano and Mayer-Gostan, 1978; Hirano, 1986).

#### *Mechanisms of Control of Prolactin Secretion*

Two control mechanisms have been suggested for prolactin secretion in teleosts: control by hypothalamic neural factors, and control directly effected by systemic factors on the prolactin cells (Nagahama *et al.*, 1975). The possibility that prolactin cells are directly influenced by plasma osmolarity has received ample attention. The activation of prolactin secretion in fish transferred from seawater to fresh water is usually accompanied or even preceded by a drop in plasma osmolarity. Moreover, not only prolactin cells *in situ*, but also prolac-

tin cells of ectopically implanted pituitary glands are stimulated by the transfer of fish from seawater to freshwater (Ball and Olivereau, 1965; Nagahama *et al.*, 1974; Wigham and Ball, 1977). The well-documented stimulation of prolactin secretion during incubation of pituitary glands in hypotonic medium is another argument that has been used in favor of plasma osmolarity as an important regulatory factor (Sage, 1968; Ingleton *et al.*, 1973; Nagahama *et al.*, 1975; Wigham *et al.*, 1977). This phenomenon has also been demonstrated for tilapia prolactin cells *in vitro* (Zambrano *et al.*, 1974; Nagahama *et al.*, 1975; Grau *et al.*, 1981).

Reduction of water pH is often followed by a drop in plasma osmolarity, caused by passive ion losses and increased osmotic water uptake (McDonald, 1983; Krout and Dunson, 1985; Wendelaar Bonga *et al.*, 1984a, b). We have suggested that the stimulation of prolactin secretion in acid water might be mediated by the decline in plasma osmolarity. However, the present data show that the activation of the prolactin cells is independent of changes in plasma osmolarity. In fact, acidification of the water to pH 4 does not necessarily lead to a reduction of plasma osmolarity: it is the *rate* of reduction of water pH, rather than the final water pH that determines the immediate effect on plasma osmolarity. Acute acidification of the water leads to more structural damage of the gill epithelium than gradual acidification, and this may explain the more severe loss of electrolytes after this treatment (Wendelaar Bonga *et al.*, 1987). This phenomenon offers the possibility of studying the effect of water pH on prolactin secretion *in situ* in the presence as well as in the absence of a decline in plasma osmolarity. The results show that the prolactin cells are activated irrespective of changes in plasma osmolarity. Apparently, the effect of water acidification on prolactin secretion is not mediated by changes in plasma osmolarity. Brewer and McKeown



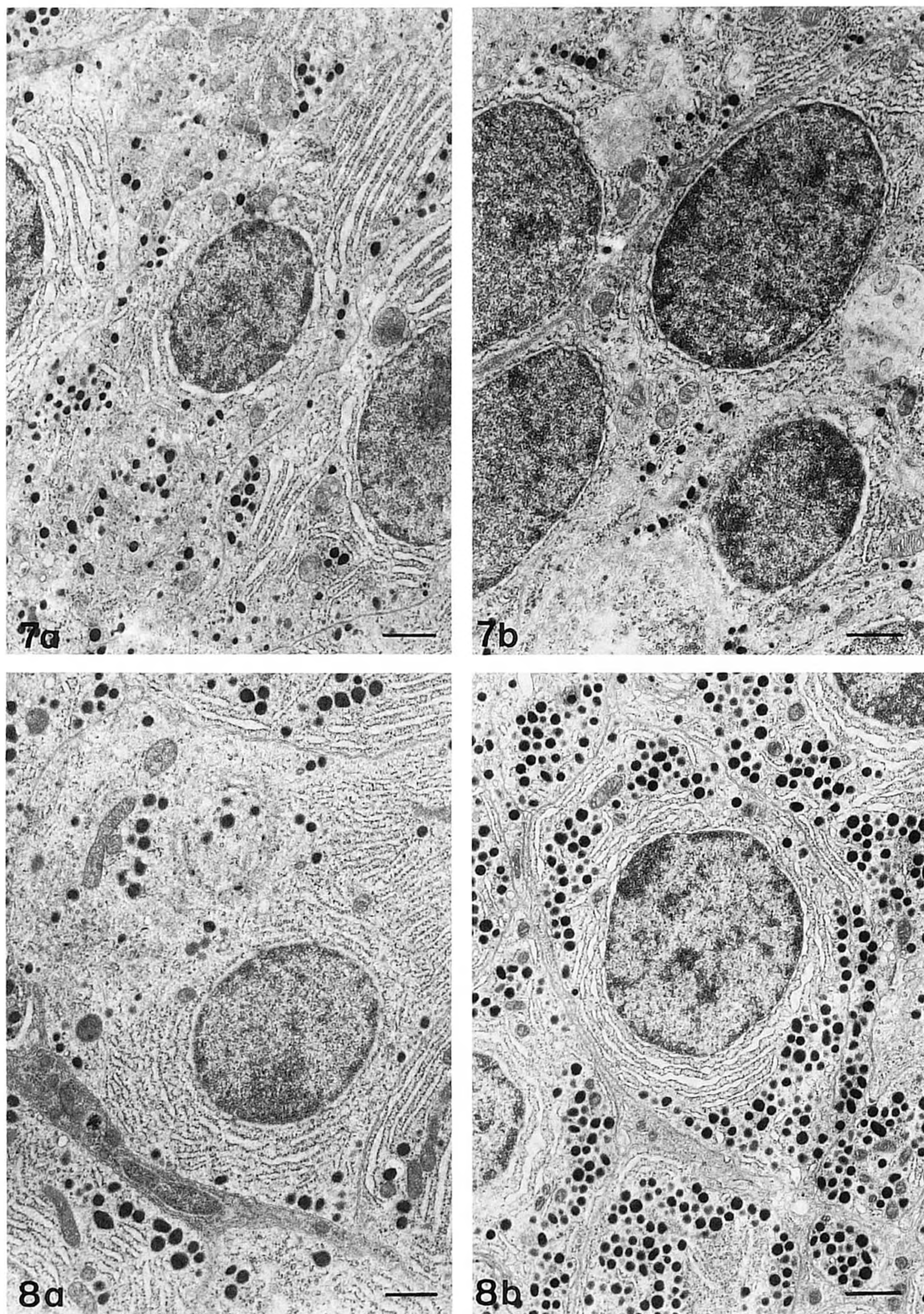


FIG. 7. Prolactin cells of *in situ* pituitary gland (a) and of pituitary implant in the same fish (b), 48 hr after acute water acidification; bars represent 1  $\mu$ m.

FIG. 8. Prolactin cells of *in situ* pituitary gland (a) and of pituitary implant in the same fish (b), 48 hr after gradual water acidification; bars represent 1  $\mu$ m.



(1980) came to the same conclusion for the effect of water osmolarity with coho salmon: stimulation of prolactin secretion after transfer of the fish from seawater to freshwater was not accompanied by changes in plasma osmolarity.

Reduction of plasma calcium or sodium levels are other factors that may stimulate prolactin secretion. After gradual water acidification, however, the prolactin cells of tilapia became activated markedly without detectable changes in plasma total calcium, total ionic calcium, or sodium. Prolactin secretion was even more stimulated in the absence of a decline in plasma osmolarity or electrolytes than in its presence. Possibly, the severe osmoregulatory stress reflected by reduced plasma electrolyte levels hampers rather than stimulates the secretory response of the prolactin cells.

How, then, is the effect of water acidification on the prolactin cells mediated? The involvement of hypothalamic control mechanisms is strongly indicated. This is concluded from the difference in the response between the *in situ* prolactin cells and the cells of ectopically implanted prolactin lobes. After gradual acidification, which does not affect plasma electrolytes, the *in situ* prolactin cells became activated, not the implanted cells. This allows the conclusion that hypothalamic connections are required for stimulation of the prolactin cells in this condition. Prolactin cells in fish, including tilapia, are under complex inhibitory and stimulatory control (Ball, 1981; Grau *et al.*, 1981, 1986; Rivas *et al.*, 1986).

In an earlier study we have shown that the inhibitory effect of elevated water calcium levels on prolactin secretion in tilapia is not mediated by increased osmolarity. Contrastingly, it was accompanied by a slight reduction of plasma osmolarity (Wendelaar Bonga *et al.*, 1985). We suggest that activation of prolactin cells by a hyposmotic stimulus, as occurs *in vitro* and in prolactin lobe implants, occurs only in the absence of intact hypothalamic control

mechanisms. The physiological significance of the hyposmotic response of the prolactin cells is unclear. Although usually less intense than in prolactin cells, this response has also been observed during incubation of other pituitary cells in fish (Ingleton *et al.*, 1973), rats (Labella *et al.*, 1975), and bovine adrenal medullary cells (Hampton and Holz, 1983). The question remains how the effect of water acidification is transmitted to the hypothalamus. The data presented in this study not only point against the possibility that changes in plasma electrolytes influence the prolactin cells directly, via the circulation, but also indirectly, via neural mechanisms. The present data are consistent with our previous suggestion (Wendelaar Bonga *et al.*, 1985) that the fish are able to perceive changes in passive ion flows and/or osmotic water flow across the integument. This suggestion was based on observations on tilapia kept in saline with an osmolarity similar to that of blood. In isoosmotic saline prolactin secretion is very low. This may be connected with the absence in isoosmotic water of substantial osmotic and ionic gradients, and thus of passive ionic net flows and of osmotic water flow across the integument. We have demonstrated that neither reduction of water calcium concentration nor acidification of the water has any effect on prolactin cell activity in isoosmotic water, although these treatments are very effective stimulants of prolactin secretion in tilapia kept in freshwater (Wendelaar Bonga *et al.*, 1984a, b, 1985). The absence of any effect of water acidification in isoosmotic saline further excludes the possibility that acidification of the blood or other body fluids stimulates prolactin secretion, since it may be assumed that the proton permeability of the integument is at least as high in isoosmotic saline as it is in freshwater (McWilliams, 1982). Mayer-Gostan and Hirano (1976) have demonstrated effects of nerve transections on osmoregulation in eels and have suggested that nerve fibers



may transmit information from branchial receptors to the brain. The presence of neural mechanisms for the perception and transmission of information on passive water and ions flows across the integument would explain our present and previous observations on the control of prolactin secretion in tilapia.

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